Cell Cycle 12:19, 3175–3183; October 1, 2013; © 2013 Landes Bioscience

CD147 regulates the expression of MCT1 and lactate export in multiple myeloma cells

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Keywords: CD147, MCT1, MCT4, lactate, multiple myeloma

Abbreviations: GEP, gene expression profiling; HMCL, human myeloma cell line; h, hours; MCT, monocarboxylate transporter; MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; PAGE, polyacrylamide gel electrophoresis; PCs, plasma cells; RT-PCR, reverse transcription polymerase chain reaction; SDS, sodium dodecyl sulfate; siRNA, small interfering RNA

Increased use of the glycolytic pathway, even in the presence of oxygen, has recently been recognized as a key characteristic of malignant cells. However, the glycolytic phenotype results in increased lactic acid production and, in order to prevent cellular acidosis, tumor cells must increase proton efflux via upregulation of pH regulators such as proton-pumps, sodium-proton exchangers, and/or monocarboxylate transporters (MCT) (e.g., MCT1, MCT4). Interestingly, expression of MCT1 and MCT4 has been previously shown to be dependent upon expression of the transmembrane glycoprotein CD147. Recently, we demonstrated that primary patient multiple myeloma (MM) cells and human MM cell lines (HMCLs) overexpress CD147. Therefore, the goal of the current study was to specifically determine if MCT1 and MCT4 were also overexpressed in MM cells. RT-PCR analysis demonstrated both primary patient MM cells and HMCLs overexpress MCT1 and MCT4 mRNA. Notably, primary MM cells or HMCLs were found to express variable levels of MCT1 and/or MCT4 at the protein level despite CD147 expression. In those HMCLs positive for MCT1 and/or MCT4 protein expression, MCT1 and/or MCT4 were found to be associated with CD147. Specific siRNA-mediated downregulation of MCT1 but not MCT4 resulted in decreased HMCL proliferation, decreased lactate export, and increased cellular media pH. However, western blot analysis revealed that downregulation of MCT1 also downregulated CD147 and vice versa despite no effect on mRNA levels. Taken together, these data demonstrate the association between MCT1 and CD147 proteins in MM cells and importance of their association for lactate export and proliferation in MM cells.

Introduction

MM is a devastating and incurable clonal plasma cell malignancy and is the second most common hematologic malignancy in the US.[1,2] All cases of MM are preceded by the premalignant stage monoclonal gammopathy of undetermined significance (MGUS). Given that MGUS is asymptomatic, clinical diagnosis is typically serendipitous; however, MGUS patients have a significant, lifelong, increased risk of progressing to MM at a rate of 1% per year.[3] Notably, many of the genetic lesions typically found in MM also exist in MGUS, which suggests that additional specific biological/genetic changes are required in order for MGUS to progress to MM.[4,5]

In this regard, we have recently demonstrated[6] that malignant PCs overexpress CD147 (also known as EMMPRIN or basigin), a transmembrane glycoprotein that belongs to the Ig superfamily. Dysregulation of CD147 expression is observed in a variety of human malignancies, and overexpression correlates with a number of biological functions that promote tumor progression (e.g., cellular proliferation, angiogenesis, MMP production) (reviewed in ref. 7). In addition, CD147 has been shown to serve as a chaperone to 2 specific members of the MCT family of proteins, which provide proton-linked transport of monocarboxylates, such as pyruvate, lactate, and ketone bodies.[8] More specifically, CD147 expression has been shown to be required for cell surface expression and function of MCT1, which is generally widely expressed, and MCT4, whose expression tends to be restricted to tissues utilizing glycolysis (e.g., muscle) but can be induced by hypoxia.[9] Both MCT1 and MCT4 demonstrate the greatest substrate specificity for L-lactate; however, lactate affinity or the K_m value is much lower for MCT1 than for MCT4, and thus, when MCT1 is expressed within a cell, it tends to be the primary regulator of lactate fluxes.[10]

Increased use of the glycolytic pathway, even in the presence of oxygen, was first noted in tumor cells by Otto Warburg in 1925.[11] However, this concept, known as the Warburg effect or aerobic glycolysis, has only recently been recognized as a fundamental feature of malignant cells. Increased use of glycolysis is

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Submitted: 04/12/2013; Revised: 08/14/2013; Accepted: 08/16/2013
http://dx.doi.org/10.4161/cc.26193

believed to result in a selective advantage for tumor cells; however, this glycolytic phenotype results in increased production of lactic acid, which must be exported in order to prevent cell death due to cellular acidosis.[12] Consequently, tumor cells upregulate a variety of proteins involved in proton export, such as proton-linked members of the MCT family of proteins (e.g., MCT1, MCT4). Overexpression of MCT1 and/or MCT4 has been reported in a variety of solid tumors; however, to date, lactate export and expression of MCT1 and MCT4 has yet to be examined in myeloma despite the fact that MM cells show evidence of both enhanced glucose uptake[13] and use of the glycolytic pathway.[14] Given that we have recently shown that MM cells overexpress CD147, in this study we focused on MCT1 and MCT4 expression levels as well as the functional consequences of lactate export in MM cells.

Results

GEP analysis of MCT1 and MCT4 expression in primary MM patient samples and HMCLs

Recently, we demonstrated that CD147 is overexpressed in MM.[6] Because it is known that CD147 can serve as a chaperone for plasma membrane expression of MCT1 and MCT4, we wished to assess expression of the latter molecules on myeloma cells. Analysis of publicly available gene expression profiling (GEP) data[15] revealed that MCT1 mRNA levels were significantly elevated in MM PCs (n = 559) vs. MGUS PCs (n = 44) ($P < 0.0001$) (**Fig. 1A**). By contrast, although MCT4 mRNA levels appear to be increased in a number of MM PCs compared with MGUS and normal PCs, overall these differences were found not to be statistically significantly (**Fig. 1B**). GEP data from our HMCLs compared with normal BMPCs and B cells revealed a similar trend in overexpression of both MCT1 and MCT4 (**Fig. 1C and D**, respectively); however, statistical analysis was not performed due to small sample size. Notably, statistical correlation studies (Spearman Rank Correlation test) performed using the publicly available Zhan et al.[15] GEP data sets for CD147, MCT1, and MCT4, did not demonstrate a correlation (Spearman r value <0.5 in all cases) between expression of these molecules at the mRNA level (data not shown). Next we wished to examine the expression levels of CD147, MCT1, and MCT4 following categorization of the GEP data into the 8 molecular MM subtypes. Use of the website Amazonia allowed us to obtain the categorized GEP data set,[15] and we performed this analysis as described. Notably, this analysis revealed statistically higher expression of CD147 in all MM subgroups when compared with normal BMPCs (**Fig. 2A**) and is therefore consistent with our prior work.[6] Likewise, MCT1 expression was found to be significantly higher in all molecular subgroups, with the exception of the CD2 subgroup (**Fig. 2B**). Of interest, expression levels of MCT1 mRNA in the PR subgroup was found to be significantly higher than in any other MM subgroup. Moreover, although our initial overall GEP analysis of MCT4 expression did not reveal significant expression differences between MM patient samples and normal PCs or MGUS samples, analysis of the GEP data following categorization into the 8 molecular MM subgroups revealed that expression of MCT4 is significantly increased in the CD1, CD2, and MF molecular subgroups when compared with normal BMPCs (**Fig. 2C**).

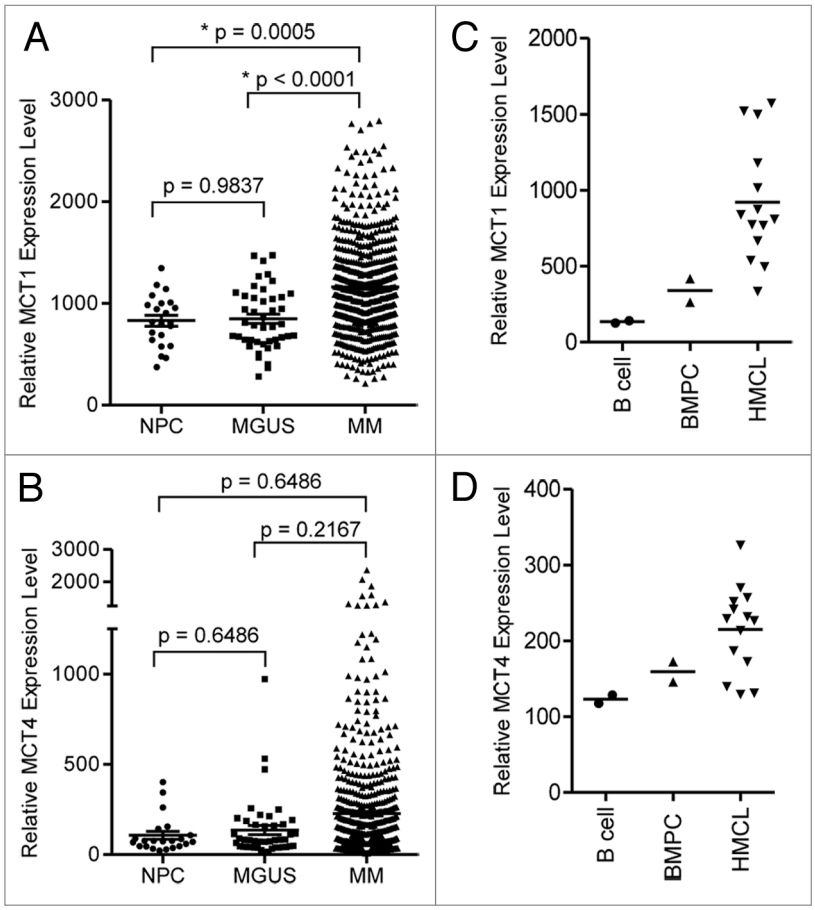

Figure 1. GEP analysis of MCT1 and MCT4 in primary MM patient samples and HMCLs. GEP analysis results for MCT1 and MCT4 (probesets 202236_s_at, 202855_s_at, respectively) in primary CD138+ samples (22 NPC, 44 MGUS, 559 MM samples) obtained from a publicly available GEP data set[15] (**A and B**, respectively) and a panel of 12 HMCLs (ALMC-1, ALMC-2, ANBL-6, DP-6, DT-6I, JMW, KAS-6/1, KP-6, MCG, MCR, RM43, and VP-6) (**C and D**, respectively).

Increased MCT1 and MCT4 mRNA and protein expression in primary MM cells and HMCLs

In order to confirm increased expression levels of MCT1 and MCT4, we next used RT-PCR and western blot analysis to confirm their expression at both the mRNA and protein levels in primary MM patient cells and HMCLs. Initial assessment of a limited number of MM patient samples compared with normal BMPCs (**Fig. 3A**) revealed increased mRNA expression of both MCT1 and MCT4. In an additional evaluation, MCT1 expression was found to be similar among never-treated and relapsed MM patient samples and HMCLs, while MCT4 expression was found to be variable among MM patient samples and generally higher in HMCLs (**Fig. 3B**). Interestingly, although all MM

patient samples and HMCLs tested demonstrated overexpression of MCT1 and MCT4 at the mRNA level, not all patient samples (**Fig. 3C**) or HMCLs (**Fig. 3D**) expressed MCT1 and MCT4 at the protein level, even though varying degrees of CD147 protein were detected in all cases. Moreover, the molecular weight of MCT1 expressed by the KP-6 HMCL was slightly higher than the molecular weight of this molecule in the other HMCLs examined. Possible explanations for the MW difference include variable MCT1 glycosylation or phosphorylation.

HMCLs and primary MM patient samples export varying degrees of lactate

Given that both MCT1 and MCT4 are capable of exporting lactate across the plasma membrane[16] and that primary MM patient samples and HMCLs were found to overexpress MCT1 and MCT4, we next wanted to determine if primary patient samples and HMCLs indeed export lactate. As shown in **Figure 4**, both primary MM patient samples (n = 8) and HMCLs (n = 5) were found to export increased levels of lactate as compared with levels exported by normal PBMCs (n = 5) and tonsillar MNCs (n = 7), which were used as controls.

MCT1, MCT4, and CD147 form a complex in HMCLs

A number of reports have demonstrated that CD147 not only serves as a chaperone for plasma membrane expression of MCT1 and MCT4, but also forms a complex with MCT1 and MCT4, which may play a role in their function as transporters of monocarboxylates, such as lactate.[17] Consequently, we next sought to determine whether either or both MCT1 and MCT4 also form a complex with CD147 in HMCLs. As shown in **Figure 5A**, immunofluorescence experiments revealed that CD147 colocalizes with MCT1 and with MCT4 in ALMC-2 cells. Subsequent immunoprecipitation experiments confirmed these findings by demonstrating that MCT1 and MCT4 could be detected via immunoblotting following immunoprecipitation of CD147 (**Fig. 5B**).

siRNA-induced downregulation of both MCT1 and CD147 decreases the proliferation of HMCLs

Previously we reported that siRNA-mediated knockdown of CD147 resulted in a significant decrease in HMCL proliferation.[6] Given that MCT1 and MCT4 form a complex with CD147, we next wanted to determine if siRNA-mediated knockdown of MCT1 and/or MCT4 would likewise affect proliferation of HMCLs. Thus, the HMCLs ALMC-2, ANBL-6, and KP-6 were transfected with either control, MCT1-, MCT4-, or CD147-specific siRNA. As shown in **Figure 6A**, using RT-PCR to screen for siRNA effectiveness, we observed significantly decreased MCT1, MCT4, or CD147 mRNA expression levels as compared with control 24 h following the second siRNA transfection (similar results were achieved in all cell lines, thus ALMC-2 results are shown as a representative HMCL in all panels). Notably, both background proliferation and IL-6 induced proliferation was significantly ($P < 0.05$) compromised following downregulation of either MCT1 or CD147 (n = 4) (**Fig. 6B**). By contrast, a significant decrease in proliferation was not observed when MCT4 was downregulated. Next we wanted to determine whether the significant decrease in proliferation following MCT1 or CD147 downregulation was due to an increase in cell apoptosis. However, as shown in **Figure 6C**, a significant increase in the number of apoptotic cells was not observed following downregulation of either MCT1 or CD147 (n = 4). The pH of the cellular media was found to be increased (n = 3) (**Fig. 6D**), while lactate export levels were decreased (n = 4) (**Fig. 6E**) following siRNA-mediated downregulation of both MCT1 and CD147 but not following MCT4 downregulation. However, neither of these findings was determined to be statistically significant. Interestingly, although RT-PCR analysis (**Fig. 6A**) demonstrated that siRNA-mediated downregulation

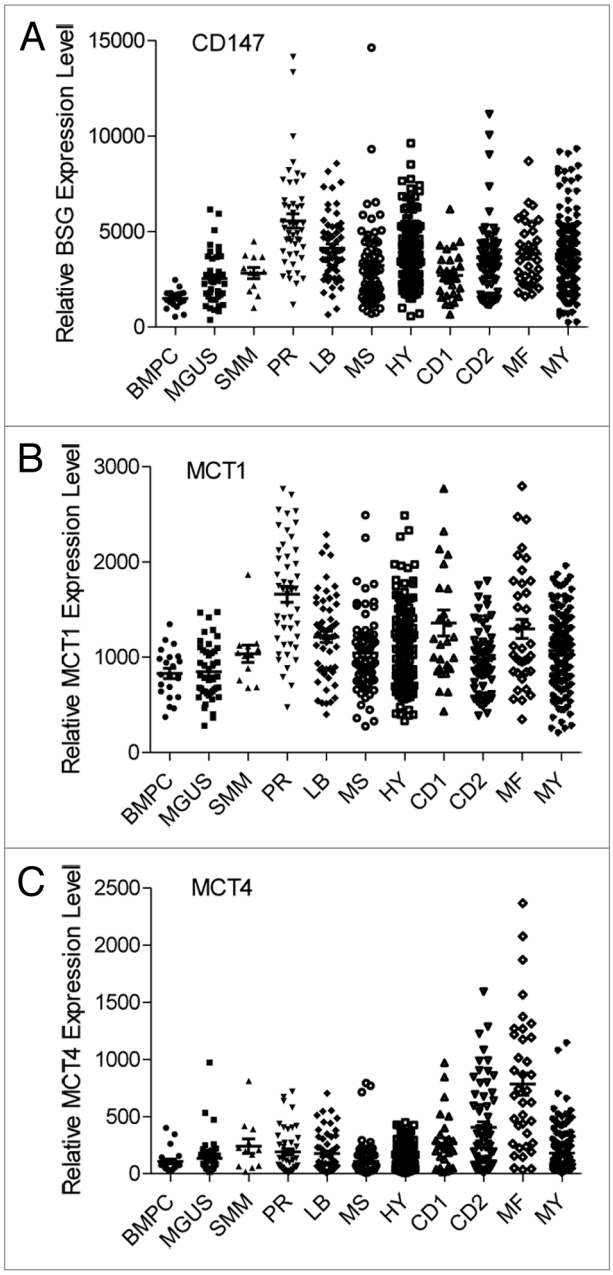

Figure 2. GEP analysis of CD147, MCT1, and MCT4 in primary MM patients following categorization into the 8 molecular subtypes of MM. Publicly available GEP data set categorized into 8 molecular subsets of MM was queried for expression levels of CD147, MCT1, and MCT4 (probesets 208677_s_at, 202236_s_at, 202855_s_at, respectively).

was specific to the targeted molecule (e.g., CD147) and did not affect mRNA levels of the other associated proteins (e.g., MCT1, MCT4), western blot analysis revealed that CD147-specific downregulation also resulted in downregulation of MCT1 at the protein level (**Fig. 6F**). MCT1-specific downregulation revealed no effect on CD147 mRNA; however, the higher glycosylated form of CD147, to which MCT1 is known to associate, was observed to be downregulated. In data not shown, these findings were further validated via flow cytometry experiments. Of note, when MCT1 was specifically downregulated, MCT4 protein levels were observed to increase. By contrast siRNA-mediated downregulation of MCT4 resulted in decreased expression of MCT4, a slight decrease in MCT1 expression, and was essentially without effect on CD147 protein expression levels.

Discussion

Expression of MCT1 and MCT4 at the plasma membrane has been shown to be dependent upon expression and association with the mature glycosylated form of CD147, which plays a role in the translocation or correct localization of these molecules.[8] In the current study, we sought to determine whether primary MM patient samples and HMCLs overexpress MCT family members 1 and 4 and whether they are found in association with CD147. Indeed, the majority of MM patient samples and HMCLs tested were found to overexpress MCT1 and MCT4 at the mRNA level; however, not all patient samples or HMCLs were found to express MCT1 and MCT4 at the protein level. Conversely, all samples and cell lines that expressed MCT1 or MCT4 expressed CD147. Similar to previously published reports MCT1 and/or

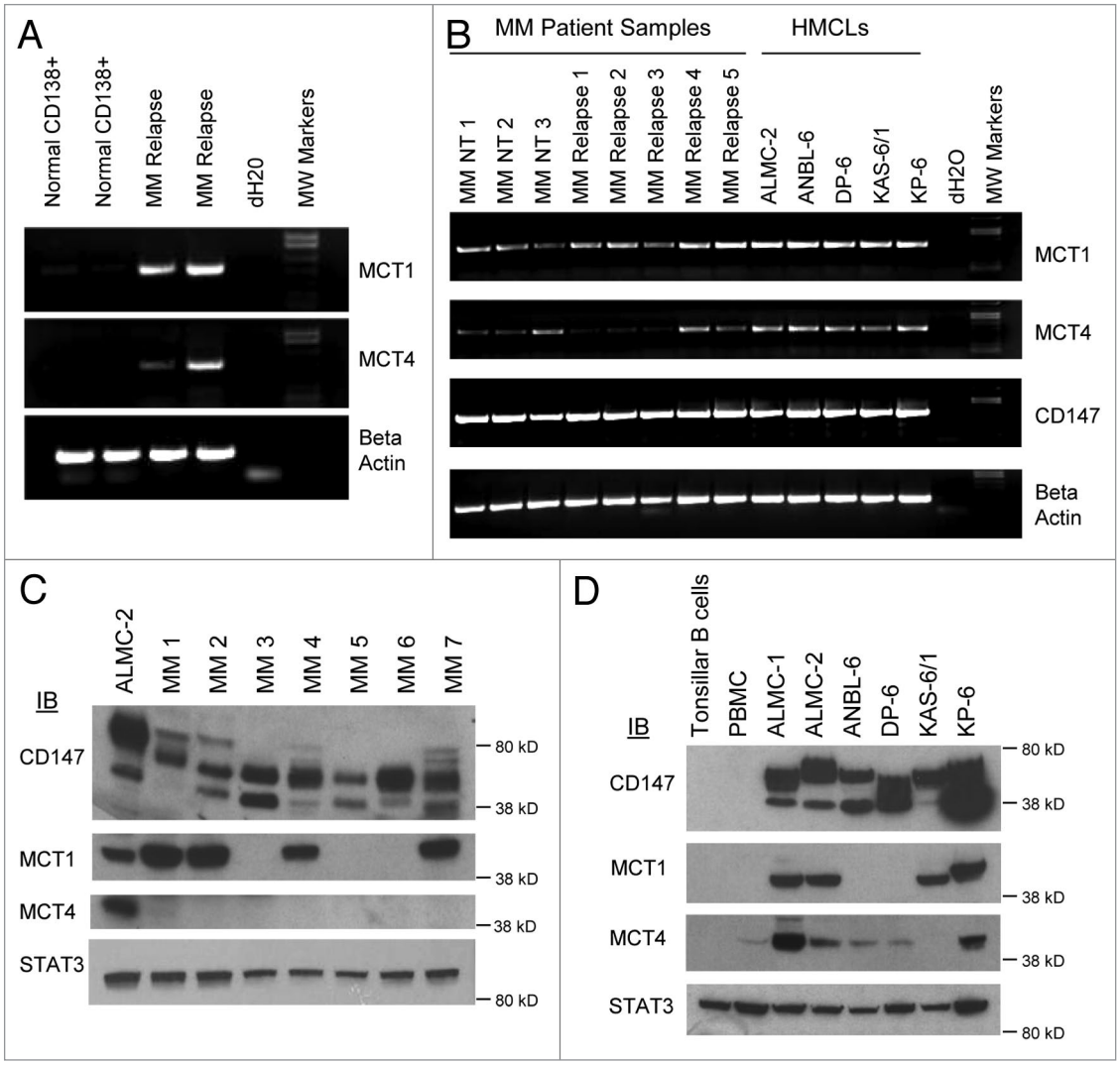

Figure 3. mRNA and protein expression of MCT1 and MCT4 in primary MM patient samples and HMCLs. (**A**) mRNA expression levels of MCT1 and MCT4 in MM patient samples vs. normal CD138+ cells. (**B**) Expanded mRNA analysis of MM patient samples and HMCLs for MCT1, MCT4, and CD147 expression. Western blot analysis of MCT1, MCT4, and CD147 protein expression in primary MM patient samples (**C**) and HMCLs (**D**). STAT3 was used as a protein loading control.

MCT4 were also found to be colocalized at the plasma membrane with CD147 in MM.

Upregulation of MCT1 and MCT4 has been demonstrated in numerous solid tumors, including colorectal, melanoma, breast, and pancreatic cancer.[18-25] However, patterns of MCT1 and MCT4 expression tend to vary among tumors, and currently, little is known about what regulates MCT1 and MCT4 expression. In general, MCT1 has been reported to be preferentially expressed in normoxic cells, while hypoxia-inducible MCT4 has been primarily reported to be expressed in glycolytic/hypoxic cells.[26] In order to determine what may influence MCT expression, several studies have recently focused on examining other factors in addition to hypoxia. For example, Boidot et al.[27] investigated the effect of extracellular lactate and p53 status on expression of MCT1 and found that addition of exogenous lactate had no effect on MCT1 expression, while tumors deficient in p53 tended to have elevated levels of MCT1.[28] The authors also found that hypoxia increased MCT1 expression in p53-deficient cells. By contrast, in preliminary studies using HMCLs possessing p53 mutations, we failed to observe this same phenomenon, which suggests that complete loss of p53 must be key to this particular response and/or that factors regulating MCT1 expression may vary from tumor to tumor. As noted above, the PR subgroup of MM expressed MCT1 mRNA at a higher level than did other subgroups of MM and normal BMPCs. Likewise, analysis of an additional publicly available GEP data set generated by Broyl et al. (2010)[29] revealed that MCT1 expression is also significantly elevated in their PR molecular MM subgroup. Thus, expression of MCT1 may be associated with the unique genetic expression pattern of the PR subgroup.

Lactate, the end product of glycolysis, is almost 99% dissociated into lactate anions and protons in biological fluids.[28] Increased use of the glycolytic pathway by tumors consequently results in: (1) an increase in intracellular lactate, which can slow the glycolytic process and (2) a decrease in intracellular pH. In order to prevent cellular acidosis and subsequent cell death, tumor cells must export protons, and one mechanism to accomplish this is increased expression of MCT1 and MCT4. In this study, we demonstrate that primary MM patient samples and HMCLs overexpressing MCT1 and/or MCT4 do indeed export lactate. Notably, the rate of lactate export tended to be greater in those cell lines expressing the highest protein levels of MCT1 and/or MCT4 by western blot, and this finding is similar to other reports in colon[18] and lung cancer.[30] Interestingly, the HMCL DP-6, which was found to express very low levels of MCT1 or MCT4 protein, was observed to export a substantial amount of lactate despite lacking significant protein expression of these 2 MCT family members. In data not shown, DP-6, as well as the HMCLs ALMC-2 and ANBL-6, were found to express low levels of the monocarboxylate family member MCT2. However, siRNA-mediated downregulation of MCT2 was found to have no effect on HMCL proliferation or lactate release.

One method to investigate whether lactate export plays an important role in MM cell biology is through the use of inhibitors. Numerous inhibitors of the MCT family of proteins have been previously described and include phloretin,

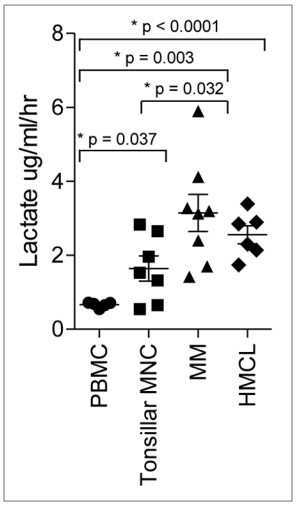

Figure 4. Measurement of lactate export in primary MM patient samples and HMCLs. Extracellular lactate levels present in the cellular media of normal PBMCs (n = 5), tonsillar MNCs (n = 7), primary MM patient samples (n = 8), and HMCLs (n = 5) were measured following a 3 h incubation in normal growth media.

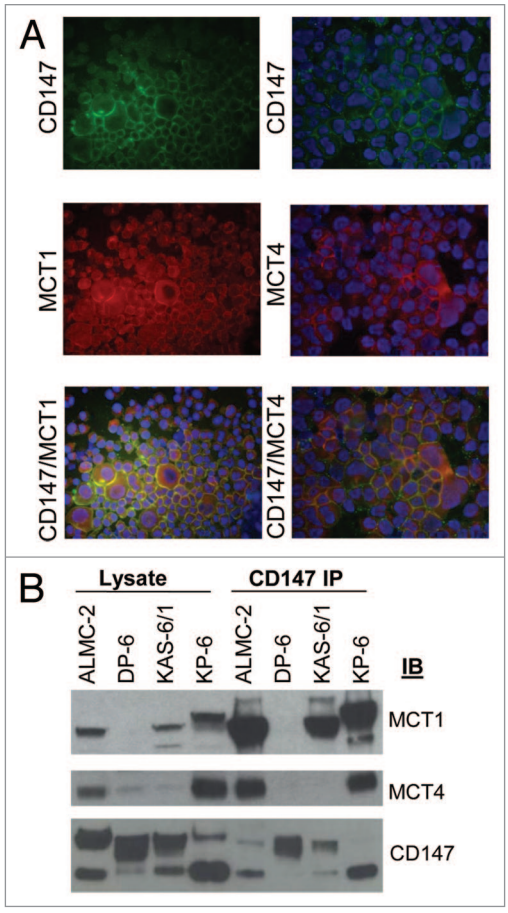

Figure 5. MCT1 and MCT4 are found in association with CD147 in HMCLs. (**A**) Immunohistochemical detection of CD147, MCT1 and MCT4 in HMCLs. ALMC-2 is shown as a representative cell line. (**B**) Detection of MCT1, MCT4, and CD147 from either 40 μg of cell lysate or following immunoprecipitation of CD147 from 500 μg of HMCL cell lysate.

quercetin, simvastatin, α-cyano-4-hydroxycinnamate (CHC), 4,4'-di-isothiocyanostilbene-2,2'-disulfonate (DIDS), and 4,4'-dibenzamidostilbene-2,2-disulfonate (DBDS).[9] However, in addition to inhibiting members of the MCT family of proteins, these inhibitors also inhibit other transport proteins (e.g., CHC also powerfully inhibits the mitochondrial pyruvate transporter), and, thus, specific conclusions from these types of experiments can be difficult. Although a specific inhibitor for MCT4 does not yet exist, a MCT1/2 specific inhibitor (AR-C155858) has been recently described.[31] Notably, Le Floch et al.[18] thoroughly demonstrated that cells which express MCT4 in addition to MCT1 or 2 are insensitive to the effects of this inhibitor. Likewise, we also

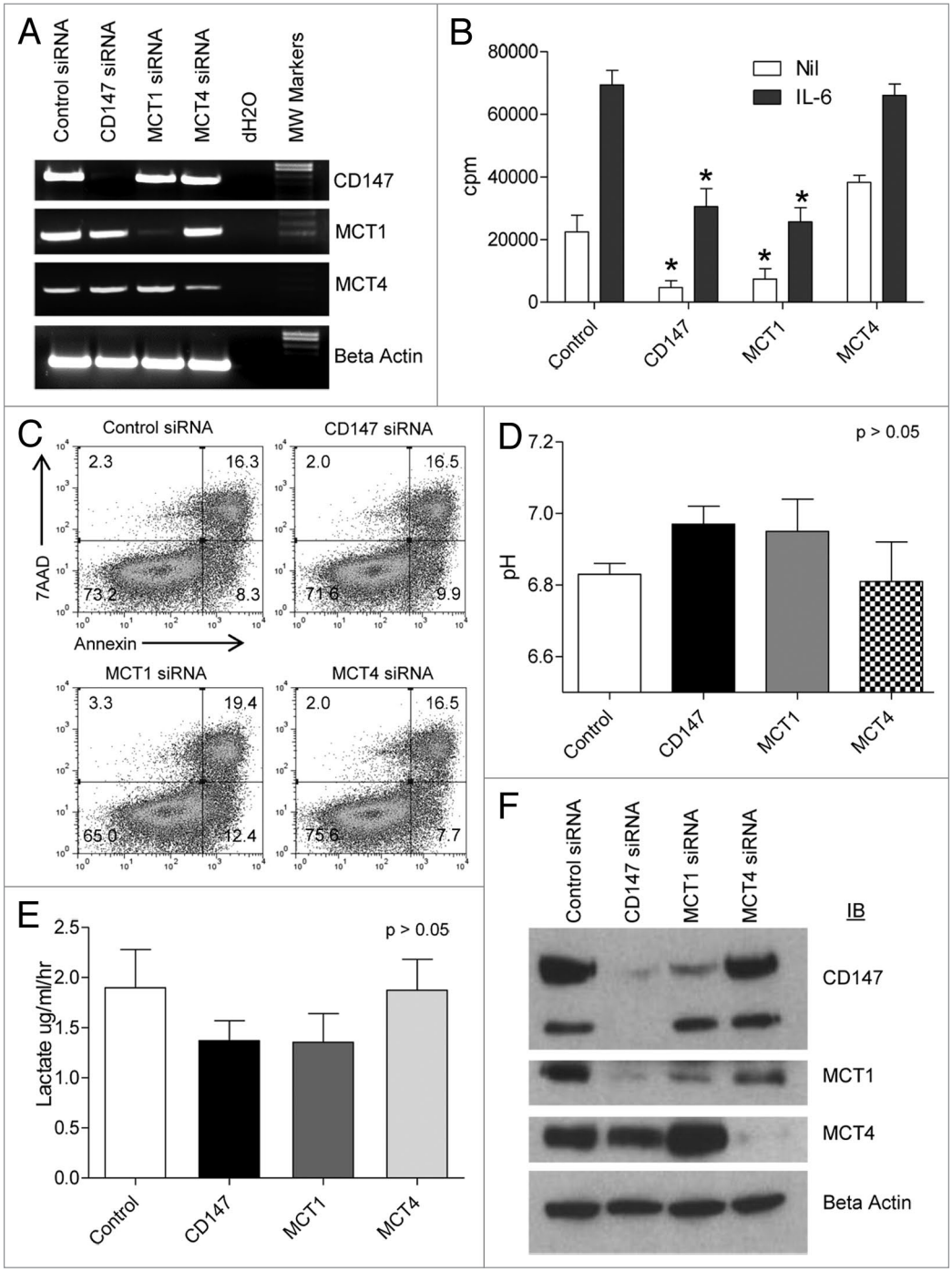

Figure 6. Downregulation of MCT1 results in decreased HMCL proliferation, decreased lactate export, and increased cellular media pH. (ALMC-2 cell line shown as a representative HMCL in all panels). (**A**) mRNA analysis of CD147, MCT1, and MCT4 expression levels 24 h following transfection of siRNA specific for CD147, MCT1, and MCT4. Seventy-two hours following transfection of siRNA specific for CD147, MCT1, and MCT4 we measured (**B**) HMCL proliferation (n = 4, * indicates $P < 0.05$); (**C**) apoptosis (n = 3); and (D) pH of cellular growth media (n = 3, $P > 0.05$). (**E**) Measurement of lactate export 24 h following transfection of siRNA specific for CD147, MCT1, and MCT4 (n = 5, $P > 0.05$). (**F**) Western blot analysis of MCT1, MCT4, and CD147 protein expression 48 h following transfection of siRNA specific for CD147, MCT1, and MCT4 (n = 3; representative result shown).

observed that our HMCLs, most of which express fairly high levels of both MCT1 and MCT4, were also relatively insensitive to AR-C155858 (**Fig. S1**). Alternatively, siRNA-mediated downregulation of MCT1 and MCT4 can also be used to investigate the role of MCT1 and MCT4. Indeed, we used an RNA-silencing approach to show a predominant role for MCT1 vs. MCT4 in lactate export in MM cells. Lactate export was also found to be linked to the expression of CD147 and MCT1, as downregulation of either molecule resulted in decreased lactate export and decreased expression of both molecules. The decrease in expression of both CD147 and MCT1, regardless of which molecule was downregulated, is most likely attributed to the close association of these 2 molecules. Interestingly, siRNA-mediated downregulation of MCT1 resulted in an increase in MCT4 expression, which we hypothesize to potentially be a compensation mechanism due to the specific downregulation of MCT1. Of note, based on the decrease in lactate export observed following MCT1 downregulation, increased MCT4 expression does not appear to compensate for the loss of MCT1, which is most likely attributed to its lower affinity for lactate. The current findings, taken together, suggest that one of the primary roles of CD147 in MM is the chaperoning of MCTs to the plasma membrane, and its particular interaction with MCT1 contributes to cellular metabolism via export of lactate. Thus, we conclude that MCT1 is an efficient lactate exporter in MM cells and plays a role in tumor growth within an acidic microenvironment.

Materials and Methods

Patient material

MM patient samples were collected as part of the routine clinical examination. Written informed consent to participate in this research study was provided by all subjects in accordance with the Declaration of Helsinki and the Mayo Clinic Institutional Review Board. MM patient samples, normal control BMPCs, and PBMCs were processed as previously reported.[32] Control B cells were purified from tonsils of patients undergoing tonsillectomy without coincident B lineage malignancy. Normal tonsillar B cells were obtained using a human B-cell enrichment kit (STEMCELL Technologies) and a RoboSep cell separator.

Cell lines, culture medium, and reagents

The HMCLs ALMC-1, ALMC-2, ANBL-6, DP-6, KAS-6/1, and KP-6 have been previously described.[33-35] The HMCLs DT-6I, JMW, MCG, RM43, and VP-6 were also developed in our laboratory but have not been previously described in the literature. All HMCLs are routinely maintained in IMDM, Glutamax (Invitrogen) supplemented with 5% fetal calf serum, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 1 ng/ml IL-6 (Novartis Pharma).

Analysis of gene expression profiling data

Publicly available gene expression profiling (GEP) data (normal PC = 22, MGUS = 44, MM = 559) generated at the University of Arkansas[15] and the Erasmus University Medical Centre[29] was analyzed for expression levels of MCT1 and MCT4 (202236_s_at, 202855_s_at, respectively). In order to evaluate expression of CD147, MCT1, and MCT4 (probesets 208677_s_at, 202236_s_at, 202855_s_at, respectively) in the 8 molecular subgroups of MM (PR, proliferative; LB, low bone disease; MS, FGFR3, WHSC1/MMSET; HY, hyperdiploid; CD1, overexpression of CCND1, ASS1, INHBE, NID2; CD2, overexpression of CCND1 and the B-cell markers CD20; VPREB3, CD79A, and BANK1; MF, MAF/MAFB; MY, myeloid), we used a website (http://amazonia.montp.inserm.fr) where categorized gene expression data of the Arkansas group are available. GEP data (Affymetrix Human U133 Plus 2.0) from our HMCLs (ALMC-1, ALMC-2, ANBL-6, DP-6, DT-6I, JMW, KAS-6/1, KP-6, MCG, MCR, RM43, and VP-6), normal B cells, and normal BMPCs were analyzed for expression of MCT1 and MCT4. Statistical analysis was performed using the Mann–Whitney test and statistical significance concluded when $P < 0.05$. Linear regression studies (Spearman Rank Correlation test) using these GEP data sets were performed to determine whether a correlation exists between expression of CD147/ MCT1, CD147/MCT4, and/or MCT1/MCT4. A Spearman correlation <0.5 was considered not well correlated; a value of 0.5–0.6 was considered a weak correlation; and a value >0.6 was considered highly correlated.

Immunofluorescence (IF) analysis

IF analysis of CD147, MCT1, and MCT4 was performed using cytospin preparations of cells mounted on glass slides. Briefly, cells were mounted on slides via centrifugation using a Thermo Shandon cytospin 2, fixed with a 4% paraformaldehyde solution for 20 min at room temperature (RT), washed 3 times with wash buffer (Dako), and permeabilized with 0.2% Triton-X in PBS for 10 min. Following permeabilization, cells were washed 3 times with wash buffer and incubated with Background Sniper (Biocare Medical) for 10 min at RT in order to reduce non-specific binding. Cells were then incubated with either antibodies to CD147 (Abcam) (1 μg/ml), MCT1, or MCT4 (both from Santa Cruz Biotechnology, Inc) for 1 h at RT in the dark, washed 3 times with PBS, and then incubated with an anti-Ig (H^+L) Ab (Southern Biotech) (1 μg/ml) for 30 min at RT in the dark, and washed 3 times with PBS. Following staining, cells were mounted with Vectashield containing DAPI (Vector Laboratories Inc). Cells were viewed using an Olympus AX 70 fluorescence microscope (Olympus Imaging America Inc). Images were acquired using an Olympus DP71 microscope digital camera equipped with Olympus DP Manager Software.

Immunoblotting

Cells (5×10^6) were lysed in lysate buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% sodium dodecyl sulfate [SDS], 1 mM EDTA, 15 mM sodium molybdate, 1 mM NaF), and lysates were cleared of insoluble material by centrifugation for 10 min at 14000 rpm. Lysates were then quantitated using Bradford reagent (Bio-Rad), and 40 μg of protein lysate were added to an equal volume of 2× SDS loading buffer, heated to 100 °C for 5 min, resolved by SDS-PAGE, and transferred to Immobilon-P membranes (Millipore). CD147, MCT1, MCT4, and β-actin were detected using antibodies to CD147 (GeneTex), MCT1, or MCT4 (Santa Cruz Biotechnology, Inc), and β-actin (Novus Biologicals, Littleton, Co). In all cases, the secondary Ab was horseradish peroxidase-conjugated polyclonal anti-mouse or anti-rabbit IgG (GE Healthcare). Immunoreactive proteins

were detected using an enhanced chemiluminescence detection reagent (Thermo Fisher Scientific Inc) and autoradiography.

RNA isolation, cDNA synthesis, and PCR

For analysis of MCT1, MCT4, and CD147 mRNA levels, total cellular RNA was extracted from HMCLs and purified PCs from MM patients as well as healthy donors using the Trizol reagent (Invitrogen). One μg of RNA was converted to cDNA using the GE Healthcare First Strand Synthesis Kit, and cDNA was amplified by PCR using the Qiagen HotStarTaq MasterMix Kit and primers specific for MCT1, MCT4, CD147, and β-actin as an internal control. The sequences of the upstream and downstream primers are as follows: MCT1 forward 5′- TTTCTTTGCG GCTTCCGTTG TTG-3′ and reverse 5′-TCAATTTACC CTTCAGCCCC ATGG-3′; MCT4 forward 5′-TTTTGCTGCT GGGCAACTTC TTCTG-3′ and reverse 5′-TCACGTTGTC TCGAAGCATG GGTTT-3′; and CD147 forward 5′- ACATCAACGA GGGGGAGACG-3′ and reverse 5′-GGCTTCAGAC AGGCAGGACA-3′. Amplification was performed in a Perkin Elmer 9600 thermocycler using the following conditions: 94 °C for 15 min and then 30 cycles of 94 °C for 30 s; 60 °C for 30 s; 72 °C for 60 s; and a final cycle of 72 °C for 10 min. Samples were electrophoresed in 1.5% agarose gels containing ethidium bromide.

siRNA transfection parameters

CD147, MCT1, and MCT4 siGenome siRNA duplexes were purchased from Dharmacon Research, Inc. Transfection of the siRNA duplexes was achieved via electroporation as previously described.[36] Briefly, cells were transfected with CD147 siRNA and subsequently plated in normal growth media. Twenty-four h after the first transfection, a second transfection was performed, and cells were plated in normal growth media. Twenty-four h following the second transfection, cells were washed twice prior to culture (2.5×10^4 cells/well in a final volume of 200 μl in 96-well flat bottom microtiter plates and 2.5×10^5 cells/well in a final volume of 2 ml in 24-well flat bottom plates). All cultures were performed in triplicate in media containing 0.5% BSA and IL-6 (1 ng/ml) for 3 d at 37 °C in the presence of 5% CO_2. At 48 h following culture commencement in the 96-well microtiter plates, cultures were pulsed with 1 μCi [^{3}H]-Tdr, and 18 h later, cells were harvested, and [^{3}H]-Tdr incorporation was assessed. Results are displayed as mean ± SEM. For cells cultured in 24-well plates, cells were counted and analyzed by flow cytometry at 24 h, 48 h, and 72 h following the second transfection.

Lactate measurement

For lactate measurement from primary MM patient samples and HMCLs, 5×10^5 cells were collected, washed 2× with PBS, resuspended in 200 μL of phenol red free IMDM containing 5% FCS and IL-6 (1 ng/ml), and plated in 96-well round-bottom plates. Following a 3 h incubation, 150 μL of media were collected, snap frozen, and analyzed at least 24 h later using an L-Lactate assay kit according to the manufacturer's protocol (Eton Bioscience). The same procedure was performed when measuring lactate 24 h following the second transfection of control, MCT1, MCT4, or CD147 siRNA. Results are displayed as mean ± SEM.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This work was supported in part by the National Institutes of Health (grants CA164232 and CA164204) and the Multiple Myeloma Research Foundation (awarded to DFJ). We would also like to thank Kimberly Henderson and Renee Tschumper for purifying the primary MM cells and normal BMPCs, respectively, and for experimental input from Dr Xiaosheng Wu and Tina Wong.

Authorship Contributions

DKW designed and performed research, analyzed data, and wrote the manuscript. BKA performed research and analyzed data. DFJ designed research, wrote and approved the manuscript.

Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/cc/article/26193

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